Feasibility Study for the Detection of Meat and Bone Meal in Animal Feed with Pyrolysis Gas Chromatography – Mass Spectrometry

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Content

1 ABSTRACT ........................................................................................................... 1

2 INTRODUCTION .................................................................................................. 1

2.1 BACKGROUND ............................................................................................... 1

2.1.1 Animal meal ............................................................................................... 2

2.2 SCOPE OF THE INVESTIGATION .................................................................. 3

2.2.1 Alternative methods .................................................................................. 4

2.2.2 Pyrolysis and analysis ................................................................................ 4

2.2.3 Curie-Point-Pyrolysis ............................................................................... 5

3 EXPERIMENTAL SET-UP ................................................................................. 5

3.1 INSTRUMENTATION ....................................................................................... 5

3.2 SAMPLING ....................................................................................................... 6

4 RESULTS ............................................................................................................. 7

5 CONCLUSION ..................................................................................................... 11

5.1 SYNOPSIS ...................................................................................................... 11

5.2 COMPARISON TO ALTERNATIVE METHODS ............................................ 12

6 REFERENCES .................................................................................................... 13
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1 Abstract
The feasibility of gas chromatography coupled to mass spectrometry (GC-MS) as rapid analytical method for the determination of meat and bone meal\(^1\) (MBM) in animal feeding stuff was evaluated. The aim of this investigation was the development of a method for detecting a contamination of 0.5% MBM in varying matrices of vegetable animal feed without the necessity of tedious sample pretreatment i.e. extraction or clean-up steps. Therefore, Curie-Point-Pyrolysis was used for sampling. The performance of the method for a potential routine screening was compared to alternative analytical techniques which are either established as routine methods or are also presently under evaluation.

2 Introduction
2.1 Background
After the first verified case of BSE 1986, current feeding of mammalian proteins to ruminants was prohibited in 1994 [1]. Legislation foresees processing/disposal measures for animal by-products (ABP) in order to minimize the risk of the spreading of BSE. Other important measures are pressure-cooking (133 °C, 3 bars, 20 minutes) of animal by-products [2] and the removal of cattle, sheep and goat specified risk material (SRM) from human and animal food chains [3]. In 2000 a complete ban on feeding of processed animal proteins to farmed animals became effective, including a ban on the use of mechanically recovered meat (MRM) from the bones of cattle, sheep and goats [4]. Therefore, the efficient control of the meat and bone meal (MBM) ban in feed is relevant to the European BSE related safety policy [5]. A comprehensive chronological list of EU legislation is published on the internet site of the European Commission [6]. Prohibited are the recycling of fallen stock and condemned animal material in animal feed, and in

\(^1\) 'Meat and bone' and 'animal meal', object of this investigation are used throughout this document for the problem of detection in animal feed, imply 'Processed Animal Proteins' (as in Decision 2000/766/EC), which cannot be fed to all animals which are kept, fattened or bred for the production of food. These are: meat and bone meal, meat meal, bone meal, blood meal, dried plasma and other blood products, hydrolysed protein, hoof meal, horn meal, poultry offal meal, feather meal, dry greaves, fishmeal, dicalcium phosphate, gelatin and any other similar products including mixtures, feedingstuffs, feed additives and premixtures containing these products.
particular intra-species recycling (the use of animal products from one species in feed for
the same species) [7,8].

However, in order to avoid the current incineration, landfill or burial, legislation
regulating waste recovery (e.g. co-incineration in power stations, cement kilns, burning
fat as fuel, use in biogas and biofertilizers, composting) is envisaged. Also, the placing of
non-SRM on the market (rendering to produce MBM and fats for use in feed, fertilizers,
pet food, cosmetic, pharmaceutical products, etc.) is under discussion, under reserve of
the safety of use and the ability to ensure the absence of SRM in products in circulation.
Suited for use in animal feeding stuff for non ruminants would then merely be material
also fit for human consumption provided an analytical technique proving the presence of
MBM in feed for ruminants can be established [9].

This investigation was considered to be complimentary of the European project
‘Stratfeed’: Strategies and methods to detect and quantify mammalian tissues in feeding
stuffs (Growth Programme, G6RD-2000-CT-00414). The objective of the ongoing
project is the development and validation of methodologies (apart from the state of the art
microscopy) to determine the presence of MBM in feed. The STRATFEED consortium is
coordinated by the Agricultural Research Centre of Gembloux in Belgium and gathers 10
partners including official laboratories, research centres, universities and one private
company from 5 EU countries.

2.1.1 Animal meal

The chemical constituents of animal meal vary considerably, depending on the rendering
process parameters and the composition in the rendered species. Animal meal used to be
fed to poultry and livestock as a source of proteins, energy and minerals. It is a by-
product of animal tissue obtained through rendering after the removal of fat. After the
(mostly incomplete) removal of fat, MBM contains bone fragments and proteins but no
intentionally added blood, hair or hide. An example of the rough composition of animal
meal is given in Table 1 [10]. Although within specifications from different sources [11],
the composition of any MDM product can vary in percentage from the stated figures.
Also, the composition of other rendering by-products prohibited as animal feed additives,
e.g. blood meal, dried plasma and other blood products may differ considerably.
Component | Content in MBM (meat meal) | Meat meal chemical analysis (aminogramm in %)
---|---|---
Dry matter | 93.0 |ASP 0.10 | THR 1.84
Protein | 50.4 (53.0) | TYR 3.14 | MBM THR 1.74
Fat | 10.0 (12.0) | GLU 4.56 | TRY 0.22
Linoleic acid | 0.36 | METH 0.66 | MBM TRY 0.27
Calcium | 10.30 | MBM METH 0.69 | GLY 7.04
Phosphorus | 5.0 | LEU 3.33 | PHEN 1.91
Ash | 28.6 (18.7) | PRO 2.87 | CYS 1.38
Sodium | 0.70 | LYS 2.82 | ISOL 1.58
Chlorine | 0.69 | MBM LYS 2.61 | VAL 2.47
Selenium | 0.25 ppm | ARG 3.84 | SER 9.60
Zinc | 93.0 ppm | HIS 1.02 | ALA 3.54

Table 1: Meat meal and meat and bone meal composition [10]

2.2 Scope of the investigation

The objective of this particular investigation was to evaluate pyrolysis gas chromatography - mass spectrometry (Py-GC-MS) as alternative method for screening animal feed. The requirements for feasibility are:

- Detection of 0.5% MBM in varying matrices of feeding stuff with a high level of confidence (low false negative results). This means the distinction from non-animal origin material, but also from fish meal, blood, scales etc.
- Method robustness: reproducibility of results and inter-laboratory transferability of data for establishment as routine screening method.
- Low cycle time, procedure cost, and user expertise. Therefore, tedious sample pre-treatment or clean-up procedures and also reagents must be avoided.

Additionally evaluated were features not necessary for a screening method e.g. the identification of species within the MBM and the appropriateness for giving legal evidence.

The distinction between MBM and milk, fat, blood, casein, gelatine, collagen, and milk products for technical uses poses a particular challenge for the analytical method. The method must also be robust against variations in the rendering process. Animal meal production usually includes a size reduction, sterilization under the principal legal stipulations (pressure cooking at 133°C, 3 bar for 20 minutes), drying usually at 140°C, and defatting by pressing. Apart from complying with minimum legal requirements for substances intended for animal feed, the material from different rendering plants can have undergone considerably different processing (e.g. in dry or wet rendering, batch or continuous processes, and the defatting method). Any analytical method is required to
perform regardless of these variations in processing parameters and other variable material properties, e.g. particle size. In particular sterilization affects the protein structure, which of course is intended and likely to be changed by the pressure-cooking process. Methods evaluating proteins or genetic material have to prove their robustness against these variations.

The aim of this feasibility study was to demonstrate that a Py-GC-MS method would be fit for the purpose and to determine the performance characteristics of the method (e.g. variability, bias, rate of false negative results). The evaluation was based on the usefulness as a screening method where suspicious samples would be further investigated by a confirmatory method.

2.2.1 Alternative methods

The state of the art and official method for the determination of animal meal in feeding stuff is to identify bone fragments with classical microscopy [7]. Alternative methods presently evaluated within the Stratfeed project are:

- Polymerase Chain Reaction (PCR), a method of creating copies of specific fragments of DNA and the subsequent identification of their origin,
- Enzyme-linked immunosorbent assay (ELISA), a technique for detecting and measuring antigens or antibodies in a solution,
- Classical microscopy visually identifying bone fragments,
- Near-infrared microscopy (NIRM) on single particles, and
- Near-infrared spectroscopy (NIRS), classifying a multi-particle sample.

2.2.2 Pyrolysis and analysis

Pyrolysis is a sample preparation procedure used for volatilizing higher molecular weight substances for investigation. It is coupled with an analytical technique: either GC-MS, mostly used for identification of peaks eluted from the GC in qualitative data, indirect analysis [12], or MS identifying the total pyrolysate or, more often, generating 'fingerprint' spectra classified by pattern recognition techniques.

Pyrolysis is the controlled thermal degradation of organic material in an inert environment, producing a complex mixture of volatile low molecular weight compounds amenable to analysis (by GC-MS, MS, etc.). Molecules cleave at specific (temperature dependent) points to form smaller, more reduced, fragments that are characteristic of the original material [13]. However, although the spectra are characteristic for the original material, they often only show fragments requiring elaborate data evaluation. If lacking a target compound the interpretation of pyrolysis products often requires sophisticated data processing techniques i.e. multivariate statistics [14].
The practical drawbacks of pyrolysis as sample preparation technique used for screening, apart from allowing only indirect analysis, are:

- The limited sample volume of only a few mg;
- The susceptibility of the reproducibility to small variations in the measurement parameters (time and temperature dependency of primary decomposition / bond scission, non equilibrium competitive processes, secondary reactions);
- The instrument to instrument transferability of results;
- The insufficient availability of mechanistic data and spectra (often results are obtained by multivariate statistics);
- Instrument contamination and transfer line condensation affecting repeatability.

However, being a fast and convenient method for volatilizing prior to analysis, pyrolysis is used in several applications. In food science, applications of pyrolysis are published for microbiological contamination (screening, species discrimination); beverages contamination and brand discrimination; sweets and sugar containing products (e.g. honey [15,16]; food processing under heat (roasting, smoke curing, Maillard reaction); and investigation of adulteration of olive oil, cocoa butter and vegetable fats [17]. However, the widest usage of pyrolysis remains in polymer science [18].

2.2.3 Curie-Point-Pyrolysis

In order to obtain reproducible molecule cleavage one method is Curie-Point-Pyrolysis. Here, rapid heating is achieved by passing radio frequency through a coil containing a ferro-magnetic sample holder up to the Curie-point of the iron-nickel alloy the sample holder is made of (inductive heating). The resulting pyrolysate expansion in a glass tube heated at 150°C in inert carrier gas is followed by the analysis (chromatographic separation and mass spectrometry detection).

3 Experimental set-up

3.1 Instrumentation

The Pyrolysis-GC-MS analyses were carried out using a Hewlett-Packard gas chromatograph (HP 5890) and mass selective detector (HP 5971 MSD) together with a GSG Curie-Point Pyromat (GSG Mess- und Analysengeräte GmbH, Germany).

Figure 1 schematically depicts the measurement set-up with and the principle of operation of the Curie-Point Pyromat. The measurement parameters are given in Table 2.
The GC-MS was operated at 250°C splitless injector temperature with a cycle of 50°C for 2 min, ramp 15°C/min, 325°C for 5 min oven temperature at 1.0 ml/min carrier gas flow. The fused silica capillary column was a 0.25 mm i.d., 30m length HP–5M, (19091S-433, Hewlett-Packard). The stationary phase film thickness was 0.25 μm. Helium was used as carrier gas. GC/MS peak identification was performed by the Hewlett-Packard Enhanced ChemStation Software Rev. B.00.00.

For pyrolysis, the sample was introduced into the pyrolysis heater in ferromagnetic beakers. The pyrolysis sample holders applied by an autosampler were connected directly to the GC-Inlet over a short transfer line heated by the Pyromat oven and the injector operated at 280°C.

The Pyromat was operated for 10s pyrolysis at 423, 590 or 764°C with 10s purging with carrier gas, each before and after the extraction.

3.2 Sampling

For the sampling of the animal meal and feeding stuff several problems had to be overcome. Firstly, a pre-study showed that the homogeneity of the sample was not sufficient for repeatable results. One reason for this problem is the limited maximum sample volume determined by the ferromagnetic pyrolysis sample holders. Only a few hundred μg can be filled into the beakers. For large batch investigation representative sampling requires an immense number of measurements, increasing with the overall size of a batch of feeding stuff under investigation. Figure 2 shows the ferromagnetic sample holders for the Pyromat together with 1g MBM as leaving the rendering plant.
Secondly, reproducible filling of the sample holders proved to be a tedious process, which had to be optimized for screening use of the method. Therefore, meat and bone meal was ground together with water prior to sampling in order to achieve optimized homogeneity of the material. 50g MBM were mixed with 75 ml of water to obtain a slurry. Grinding was performed with a Buechi mixer for 3 min. Then, the homogenized slurry was applied to ferromagnetic wires and left for drying overnight, removing the water again. The wire was weighed before and after the application of the sample slurry. The material thus subjected to pyrolsation was 0.5 mg, less than the maximum filling of a beaker, but homogenous. The homogenization resulting from this procedure made sample introduction easier and enabled more representative measurements. However, the reproducibility of measurements remained unsatisfactory and the standard deviation exceeded 50% for the overall spectra and individual component content.

4 Results

Meat and bone meal batches from well characterized and controlled production processes were investigated. It was observed that the variation in temperature of the pressure-cooking process from 128°C to 133°C was not detectable from the resulting spectra. Pyrolysis was used for volatilizing the MBM for investigation, i.e. as extraction technique. Pyrolysis temperature variation showed that 740°C or 764°C were the optimal temperatures for the investigation. At lower temperatures, e.g. 423°C or 590°C, the yield and therefore the detection limit decreased. At temperatures higher than 764°C the increased cleavage of potential target substances of higher molecular weight into fragments difficult to attribute, proved unfavorable.

From over 30 analyses of MBM under the conditions described several compounds representative for the material were identified and their abundance was compared to the content in animal feed of vegetable origin. 34 compounds abundant in significantly
different concentration in MBM and vegetable samples and at the same time constituting more than 0.5% of the chromatogram signal area, i.e. sample concentration, were identified by mass spectrometry. However, from the identified substances only those which signify the presence of MBM in a matrix of vegetable feed would be suitable as target analytes. Figure 3 shows the abundance of 25 of the compounds identified.

![Diagram showing abundance of compounds in MBM and vegetable samples.](image)

**Figure 3:** Compounds contained in 3 MBM and 3 vegetable samples (pyrolysis at 740°C).

In Figure 3 the content of the compounds in the spectra of 3 MBM and 3 vegetable feed samples each were averaged and the standard deviation for the substances is shown as error bars for the respective compound. Figure 3 shows that the reproducibility of the measurements is poor. For most substances the abundance is not specific and sufficient for the determination of sample origin. However, the abundance of pyrrole and its derivates and the exclusive occurrence of cholesterol in MBM were considered for further investigation.

Figure 4 shows the chromatograms of three MBM samples subjected to pyrolysis at 740°C under the conditions described in Table 2. The chromatograms display low reproducibility and a large number of compounds resulting from the pyrolysis. The latter is due to the principle of pyrolysis generating more reduced cleavage products from larger molecules.

However, most of the fragments are not characteristic of the original material. Although many compounds could be identified by the mass spectra of their peaks, the peaks of the
chromatogram could not be attributed to a target compound of the original material with the exception of cholesterol at 28.7 min. retention time (RT).

If no target compound can be found, the interpretation of pyrolysis products can be performed using data processing techniques i.e. multivariate statistics. However, explorative multivariate statistics, e.g. Principle Component Analysis (PCA) visualize data for classification by emphasizing variance. If the reproducibility of measurements of the same sample as seen in Figure 4 is low, the achievable classification is insufficient for quantitative determination of contamination by MBM with the required detection limit of 0.5%. A PCA performed with the entire chromatograms of 10 samples did not yield a satisfactory discrimination of MBM and vegetable samples.

Figure 4: Chromatograms of three MBM samples (pyrolysis at 740°C)

One reason for the poor reproducibility displayed in Figure 3 and Figure 4 was the contamination of the instrument and especially the transfer line by condensation of pyrolysate which affected repeatability. MBM is a complex product with a multitude of compounds generated by pyrolysis. An inherent problem of pyrolysis is that the temperature of decomposition, here 740°C, is higher than the temperature of column inlet and column itself (see Table 2). Due to this, the instrument had to be repeatedly cleaned during the investigation. Figure 4 shows the sample holder and the glass tube surrounding the sample during pyrolysis before (left) and after (right) pyrolysis of MBM at 740°C.
Cholesterol could be clearly identified in the measurements of MBM, also the cholesterol signal is in a region of the chromatogram (RT 28.7 min) where there is little interference from any constituents of samples free of animal originate material. With identification by the mass spectrum, cholesterol content can be quantitatively correlated to MBM content. Using cholesterol as target compound, a theoretical detection limit of 1% MBM in animal feed appears achievable.

Figure 6 shows the mean of 3 MBM chromatograms together with the chromatogram of animal feed containing no substances of animal origin.

![Chromatogram](image)

**Figure 6: Chromatograms of vegetable feeding stuff and averaged MBM (pyrolysis at 740°C).**

Unfortunately, cholesterol cannot be used as a marker for MBM content as it is contained also in other potential additive materials not implicated by the ban, especially tallow and lard. Note that although the spectra of MBM and vegetable animal feed are clearly different with the variation in spectra of identical composition, multivariate statistics are not robust enough for discrimination of minor contaminations as required. Moreover, although ample target analytes for vegetable animal feed components are pervasive, a definite marker for MBM content could not be identified during this investigation.

One potentially suitable indicator for MBM content is hydroxyproline. Hydroxyproline is a nonessential amino acid, produced from proline in the mammalian liver for collagen buildup. 3- and 4-hydroxyproline is prevalent in collagen. Most collagens contain 10-15% of hydroxyproline, so the hydroxyproline content of a sample is an accurate measure of the collagen content [19]. Figure 7 shows the chromatogram of decomposition products of pure hydroxyproline by pyrolysis at 740°C.
Pyrolysis of hydroxyproline leads to a reduction to pyrrole (R=H) and its derivatives. Also here, as indicated in Figure 3 and Figure 6, the decomposition products are not specific and sufficient for the detection of MBM content.

5 Conclusion

5.1 Synopsis

Pyrolysis of a slurry at 740°C yields the best results. The classification for MBM content in the complex matrix animal feed is possible. However, within the scope of this preliminary feasibility study, the limit of detection can be only estimated. Taking into account only the limited number of measurements done the detection of MBM content less than 5% could not be proven although improvements of method sensitivity seem possible.

In summary, pyrolysis-GC-MS analysis was found to be unsuitable for use as a screening method for MBM in animal feed. Several reasons for this could be identified. Firstly, pyrolysis does not result in products of thermal degradation that are specific to MBM content. Substances marking down material of animal origin could be identified from pyrolysis-GC-MS spectra but no marker for the specific material of interest could be found. Secondly, the reproducibility of the technique was found to be insufficient for the determination of MBM content in a variable matrix in the required concentration range of 0.5%. Pyrolysis-GC-MS analysis allows to ascertain MBM contamination in high concentrations. Additional benefits, e.g. species identification, appear more than unlikely. Also, the technique is not suited as quantitative method. Thirdly, the inhomogeneity of
animal feed and the chemical complexity of the material inhibit direct sampling and lead to problems with instrument contamination. Thus the cycle times for pyrolysis of animal feed have no dispositive edge over alternative techniques.

### 5.2 Comparison to alternative methods

Table 3 gives a preliminary overview on the applicability of the five methods that are currently under investigation within the Stratfeed shared cost action project for the determination of meat and bone meal in animal feed. Suitability of the method is indicated by '+' or '-', respectively. If the relevant data is not available or the method has not been validated yet this is indicated by a question mark.

<table>
<thead>
<tr>
<th>Method</th>
<th>Pyrolysis-GC-MS</th>
<th>PCR / Real time PCR</th>
<th>Microscopy (bones)</th>
<th>NIR (microscopy)</th>
<th>Elisa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Classification for MBM content</td>
<td>+/-</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Limit of detection</td>
<td>5%?</td>
<td>0.1%</td>
<td>0.1%</td>
<td>0.1%</td>
<td>0.1</td>
</tr>
<tr>
<td>Method robustness, e.g. mis-classification of heat treated material</td>
<td>++?</td>
<td>+ - ?</td>
<td>++</td>
<td>++</td>
<td>+ -</td>
</tr>
<tr>
<td>Reagents / costs</td>
<td>+/-</td>
<td>--</td>
<td>--</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Discrimination of fat / milk / blood</td>
<td>--</td>
<td>+/-</td>
<td>++</td>
<td>+/-</td>
<td>++</td>
</tr>
<tr>
<td>Species identification</td>
<td>--</td>
<td>++</td>
<td>--</td>
<td>--</td>
<td>++</td>
</tr>
<tr>
<td>Instrument to instrument data transferability</td>
<td>--?</td>
<td>+</td>
<td>++</td>
<td>?</td>
<td>? +</td>
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<td>--</td>
<td>+/-</td>
<td>+</td>
<td>--</td>
</tr>
<tr>
<td>Legal evidence</td>
<td>--</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>--</td>
</tr>
<tr>
<td>Suitability as screening method (cycle time, procedure costs, user expertise)</td>
<td>--</td>
<td>--</td>
<td>+/-</td>
<td>++?</td>
<td>++</td>
</tr>
</tbody>
</table>

Table 3: Comparison of methods currently under investigation for meat and bone meal determination in animal feed [20].
6 References

http://europa.eu.int/comm/food/fs/bse/legislation_en.html#Chronological%20list
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